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JOINT OIHP/WHO STUDY-GROUP
ON CHOLERA

Report on the Third Session

New Delhi, 18-19 November 1949

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JOINT OIHP/WHO STUDY-GROUP ON CHOLERA

Third Session

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The report on the third session of this study-group was originally issued in mimeographed form as document WHO/Cholera/6, 30 November 1949.

JOINT OIHP/WHO STUDY-GROUP ON CHOLERA

Report on the Third Session¹

The members of the Joint OIHP/WHO Study-Group on Cholera arrived in India on 31 October 1949 and visited the following places: Bombay; Madras; Trichinopoly; field investigation areas in Tanjore, and neighbouring villages in the Tanjore district; Calcutta and the rural areas of Diamond Harbour; Dacca and some adjacent rural areas in Pakistan.

The study-group visited medical institutions of interest in each of these places, had interviews with the health ministers and administrative medical officers, and held discussions with cholera research workers.

During their tour, members were able to see cholera cases in hospitals and in the rural areas and to inspect local arrangements as regards water-supplies, drainage, disposal of sewage in relation to the bathing ghats (riverside bathing-places), the bustees (slums), and river and canal systems. They saw the environmental conditions and studied the habits of the people both in urban and rural surroundings.

In the Tanjore District the study-group made an exhaustive tour of the rural area where cholera investigation operations under the auspices of the Indian Research Fund Association (IRFA) have been initiated. Members were able to visit the actual homes in the villages and to see the people in their local surroundings. They visited cholera patients in adjacent villages. The study-group was impressed by the co-operation shown by the population of the area and their desire to improve their sanitary conditions. The study-group spent considerable time at the field laboratory in Trichinopoly where specimens from the area under investigation are studied. The laboratory was found to be well housed, and adequately staffed and equipped for undertaking mass bacteriological examinations of stools and water.

¹ The Executive Board, at its fifth session, adopted the following resolution:

The Executive Board

(1) NOTES the report of the Joint OIHP/WHO Study-Group on Cholera on its third session; and

(2) AUTHORIZES its publication.

(*Off. Rec. World Hlth Org.* 25, 6)

From 15 to 17 November the study-group met in joint session with the Cholera Advisory Committee of the Indian Research Fund Association at New Delhi. At the joint session, in addition to the members of the study-group and the members of the Cholera Advisory Committee of the Indian Research Fund Association, were present administrative medical officers and directors of public health from cholera-infected provinces, research workers, and other scientific workers.² Papers were presented and discussions took place on the following subjects:

- (1) Statistical analysis of cholera incidence in the Indian peninsula for the last 45-year period, to determine the endemic foci of infection.
- (2) Statistical study of cholera incidence in India and Pakistan to assess the trend of the disease.
- (3) Fairs and pilgrimages and their influence on the spread of cholera.
- (4) Examination of water sources; study of carrier state; viability of cholera vibrio on and in fruits, food, and flies; laboratory studies with reference to smooth (S) and rough (R) forms of the cholera vibrio; influence of sulfonamides on carrier state; agglutination response; immuno-chemical studies; standardization of cholera diagnostic procedures; and, finally, control measures.

The Joint OIHP/WHO Study-Group on Cholera held its third session from 18 to 19 November 1949 at the WHO Regional Office for South-East Asia, New Delhi, India. Dr. C. G. Pandit was elected Chairman.

1. Quarantine Aspects of the Cholera Problem, and Items Referred to the Study-Group by the WHO Expert Committee on International Epidemiology and Quarantine

1.1 Persistence of cholera vibrios in convalescents and contacts

It was noted that, although further work on this subject was in progress, no new points had come to light so far. The study-group recommend that work be continued.

1.2 Viability of Vibrio cholerae on and in fruits, food, and flies

Investigations undertaken on fruits, vegetables, and flies for the presence of cholera vibrio had proved inconclusive. The importance of examining these foodstuffs and flies collected from inside infected premises was stressed. Following the Japanese work on infected fish and the dissemination of cholera through that source, and in view of the possibility that fish infected with cholera vibrios could infect water-supplies, further

² See Annex 4, page 21.

investigations on the role of fish and other aquatic fauna and their potential danger in infecting water-supplies should be made.

1.3 *Observations on the possibility of subclinical and inapparent cases constituting a link between successive cholera outbreaks in an endemic area*

The study-group noted that, in accordance with the recommendations made at the second session of the study-group,³ an investigation into the possibility of subclinical and inapparent cases constituting a link between successive cholera outbreaks in an endemic area had been started recently in the Tanjore District under the auspices of the Indian Research Fund Association and with the help of the Office International d'Hygiène Publique (OIHP). The study area selected comprises a group of 30 villages with a population of approximately 60,000. The investigation in this area will be continued for at least two cholera seasons, during which period a close study will be made of all relevant factors in humans, such as the role of subclinical and inapparent cases, contacts, carriers, and convalescents, as well as of environmental factors such as water-supplies. The field laboratory established at Trichinopoly is undertaking this work, which has just started, so that results will not be available for some time yet.

1.4 *Standard procedure for the diagnosis of cholera*

The method described in a note prepared by Ahuja, Krishnan, Pandit, & Venkatraman on the laboratory diagnosis of cholera⁴ is recommended as a standard method for the diagnosis of cholera vibrio.

1.5 *Influence of sulfonamides upon vibrio excretion of convalescents and contacts*

It was noted that work was in progress on this subject. The preliminary data so far available are insufficient for definite conclusions to be drawn.

1.6 *Bandi's test*

The study-group at its second session had recommended a comparative study of the results obtained with Bandi's test and with the classical methods;⁵ research is in progress.

1.7 *Agglutination response for the retrospective diagnosis of cholera*

A paper by Krishnan & Dutta on the retrospective diagnosis of cholera through study of agglutination response following anticholera inoculation

³ *Off. Rec. World Hlth Org.* 19, 25

⁴ See Annex 1, page 10.

⁵ *Off. Rec. World Hlth Org.* 19, 25

was presented at the joint meeting with the IRFA.⁶ The study-group considers that this work is likely to prove valuable:

- (1) as a means of retrospective diagnosis of cholera;
 - (2) in investigating immunity in the population of an endemic area.
- This investigation may also be useful in assessing the value of anticholera vaccination.

The study-group recommends that this work be continued and that a large-scale investigation of normal sera from non-endemic areas, such as non-cholera areas of West Pakistan, be undertaken in India and in other regions.

1.8 Vaccination

A note by Dr. Melville Mackenzie, prepared for the second session of the Expert Committee on International Epidemiology and Quarantine, on the subject of inoculation against cholera⁷ was referred to the study-group. The study-group reiterates the opinion expressed during its first session⁸ that inoculation is of value and must be taken into account in applying quarantine measures. For this purpose, it advises two injections at a week's interval. Booster doses, if required within six months, may consist of one injection, beyond which period two injections will be necessary. The vaccine employed must conform to the standard laid down by WHO.

The study-group also draws the attention of the Expert Committee on International Epidemiology and Quarantine to its view, previously expressed as follows:

"In view of the relative character of the immunity conferred by vaccination, vaccinated persons should not be exempted from all measures of control by the international quarantine regulations. Sanitary "surveillance" now provided in the international sanitary convention is, however, considered as an adequate protective measure."⁹

1.9 Paracholera

The study-group was of the opinion that paracholera was not a disease for which quarantine measures need be considered.

2. Laboratory Studies

2.1 S and R forms of cholera vibrio

The study-group noted with interest the S to R mutation of the cholera vibrio in the stools of convalescents described by Dr. Bruce White

⁶ See Annex 2, page 15.

⁷ Unpublished working document WHO/Epid/25.

⁸ *Off. Rec. World Hlth Org.* 11, 16

⁹ *Off. Rec. World Hlth Org.* 11, 16

and considered it desirable to investigate this phenomenon further to confirm this observation. A preliminary note on R to S variation of *V. cholerae* in the presence of antiphage serum, by Dr. Doorenbos and Cossery Bey,¹⁰ was presented to the study-group by Sir Aly Shousha, Pasha. The study-group considered that further work in this direction should be undertaken to confirm results.

2.2 *Immuno-chemical studies on V. cholerae*

The study-group noted with great interest the work resulting in the isolation from the cholera vibrio of a polysaccharide fraction possessing antigenic properties. These researches may open new fields for the development of a more potent cholera vaccine.

2.3 *Toxin*

The study-group noted with particular interest the recently described tests with cholera toxin filtrates. The application of these tests may lead to a better understanding of the pathogenesis and immunology of cholera.

3. Endemicity

3.1 *Geographical distribution of cholera endemic areas*

The results of statistical studies presented by Dr. S. Swaroop¹¹ seemed to indicate that in addition to East and West Bengal other deltaic areas, such as the Cauvery delta in the Madras Province and the Mahanadi delta in Orissa, are also endemic. As regards China, the evidence appears to be against its being a true endemic area. In Indochina, the southern delta (Cochin-China and southern Cambodia) has generally been considered as an endemic area on the basis of data obtained for the last three years. It is, however, noted that during the first nine months of this year this area has been relatively free from cholera.

3.2 *Factors governing endemicity of cholera in India*

The following conditions seemed to favour endemicity of cholera :

- (1) deltaic conditions ;
- (2) high density of population ;
- (3) high humidity ;
- (4) abundance of an uncontrolled water-supply ;
- (5) salinity and high organic content of water.

¹⁰ See Annex 3, page 18.

¹¹ Unpublished working document WHO/Cholera/5

It was, however, noted that certain regions where many of the above conditions existed were not endemic although subject to periodic epidemic invasions. It appeared to the study-group that a study of different regions where such conditions existed might throw further light on the probable and relative importance of the above conditions. For this purpose, it recommends that comparative studies in similar deltaic regions outside India be undertaken.

3.3 *Pilgrimages and fairs*

There appeared to be no relation between localization of fairs and endemicity of cholera in an area. Congregation of the people was accepted as an important factor in the spread of infection not only in these gatherings but also in the collective movement of labour.

3.4 *Presence of cholera vibrios in tanks and other natural water-supplies*

Investigations recently undertaken in India relating to the isolation of cholera vibrios from water-supplies have shown a number of positive results from Calcutta waters, in marked contrast to water examinations in other areas. This may be due to the Kieselguhr technique now being used by Indian workers.

4. WHO Cholera-Control Programme for 1950

The study-group noted with satisfaction the following decision of the Second World Health Assembly :

“ Cholera is a disease for which true eradication—that is to say, world eradication—can and must be aimed at . . .

The immediate objective is gradually to clean up the truly endemic area, after a preliminary delineation of this area and a study of the factors of endemicity . . . have been made.”¹²

The study-group recommends the formation of field teams, as envisaged by the Second Health Assembly. In view of the recent statistical studies indicating the existence of endemic areas in India outside West Bengal, particularly in the deltaic area of Madras, and of the organization recently set up in that province to investigate cholera endemicity, the study-group recommends that the field team for India should be assigned to this area. As regards the second field team, the study-group recommends the selection of a suitable area in the East Bengal province of Pakistan.

¹² *Off. Rec. World Hlth Org.* 18, 110

Realizing that environmental sanitation factors will play a prominent role in cholera-control programmes, the study-group endorsed the importance of close co-operation with the WHO Section on Environmental Sanitation at all stages of the operation.

In demonstrating control methods and developing techniques with the ultimate objective of cholera eradication, the study-group would lay special emphasis on the following points :

(1) Provision of pure water in abundant quantities in order to obviate the necessity of using uncontrolled water. For this purpose it would be essential to work out effective and economical methods.

(2) Development of suitable and economical excreta disposal methods with reference to family dwellings.

(3) Health education of the affected population to ensure co-operation of the local people and utilization of the facilities provided. Co-operation with the WHO Section on Health Education of the Public would be necessary in this direction.

A period of study and survey would be necessary before the undertaking of actual field operations.

The study-group was of the opinion that this problem required urgent attention and that, therefore, provision should be made for the implementation of the WHO cholera programme as soon as possible. The organization of the teams should be proceeded with immediately.

Annex 1

LABORATORY DIAGNOSIS OF CHOLERA

Bacteriological Procedures¹

1. The bacteriological diagnosis of cholera includes the isolation of *V. cholerae* from the stool or vomit of a case under investigation, and the identification of the vibrio by morphological, cultural, biochemical, and serological procedures. The isolation of *V. cholerae* may be very easy or remarkably difficult depending upon whether one is dealing with an established case of cholera in its acute stage during an epidemic or with a convalescent or a contact some time after the subsidence of the epidemic. Likewise, the identification of the organism can more readily be assumed from a few simple tests, including a test of agglutinability with a specific 'O' agglutinating serum, during an epidemic of cholera, but should be based upon a complete examination in the case of the occurrence of a case of cholera-like disease during non-epidemic times or at the beginning of an epidemic and more especially in areas usually free from cholera. Various procedures have been followed in the isolation and identification of *V. cholerae* with more or less satisfactory results; those preferred by the authors after more thorough investigation are presented here.

2. Collection of specimen: The isolation of *V. cholerae* from a suspected case is most readily made from a specimen of freshly passed stool or one obtained by the insertion of a rectal swab the handle of which is protected by a piece of rubber beyond the anal sphincter. If facilities for adequate bacteriological examination are available locally the material is plated directly on a suitable solid medium. Selective media mentioned later can bear a comparatively heavy inoculum. If, however, a delay of some hours is anticipated, or if the specimen has to be sent to a distant laboratory by post, a small portion of the stool is placed in a bottle containing 10 ml of a preserving medium of potassium chloride solution, boric acid, caustic soda, and sea salt.² In the case of a specimen obtained by rectal swabbing, the swab is immersed in the preserving medium and squeezed out against the side of the bottle. In the case of examination of convalescents or

¹ Note submitted by: Lt-Col. M. L. Ahuja, Director, Central Research Institute, Kasauli, India; Dr. K. V. Krishnan, Professor of Microbiology, All-India Institute of Hygiene and Public Health, Calcutta, India; Dr. S. R. Pandit, Director, Institut Pasteur, Shillong, India; and Dr. K. V. Venkatraman, Director, King Institute, Guindy, Madras, India.

² See Appendix 1, page 13.

contacts it is more satisfactory to add a specimen of stool (about 1-3 g) to the preserving medium. Specimens of vomit, or shreds of clothing soiled with stool or vomit of a case of cholera, may similarly be added to the preserving medium.

3. Microscopic examination of a stained film (stained with diluted carbolfuchsin) and a wet film from washed flakes of mucus present in the rice-water stool of a case of cholera, revealing the presence of numerous actively motile vibrios, may form the basis of a provisional diagnosis of cholera. Too much reliance should not, however, be placed on morphological examination alone; the most characteristic appearances are found only in those cases where clinically a diagnosis of cholera is almost certain, and non-choleraic vibrios indistinguishable morphologically from true *V. cholerae* abound in almost all the water in the plains of India and are to be found mixed with stool specimens.

4. In the cultural examination of a specimen for the isolation of *V. cholera* from an acute case, direct plating of fresh material or material held in the preserving medium is preferred to plating after enrichment. Either plates of alkaline nutrient agar, or a differential medium such as Aronson's medium, or a modified Wilson and Riley's solid medium may be used.³ The superiority of the results obtained with direct plating over those obtained with preliminary enrichment followed by plating has been pointed out by Pandit, who examined 430 specimens of stool samples simultaneously by both the methods, using in each case alkaline agar, Aronson medium, and the modified Wilson and Riley solid medium, for plating; he found that 96% of the positives were picked out by direct plating, whereas only 89% were picked out by the procedure utilizing enrichment followed by plating. Our experience has also been similar. Of the plating media mentioned above, both the Aronson medium and the modified Wilson and Riley's medium have an inhibitory effect on many organisms other than vibrios, the latter medium being superior to the former in that it inhibits also the El Tor vibrio to some extent and many inagglutinable vibrios, though not all. While generally reliable, certain batches of Aronson's medium turn out to be poorly supportive of the growth of *V. cholerae*.

5. In the case, however, of the examination of stools of convalescents and contacts, a preliminary enrichment is desirable. Read's modification of Wilson and Blair's fluid enrichment medium has been most useful in our experience.⁴ This is particularly valuable when vibrios are very scanty in the stool. The fluid enrichment medium is plated with 10 ml of the preserving medium containing the entire stool specimen, and a few loopfuls of

³ See Appendix 2, page 14.

⁴ For composition, see Appendix 3, page 14.

the enriched culture are subcultured on Riley's solid medium after 18-20 hours' incubation. The necessity for the readjustment of the pH of culture during enrichment after about 4-6 hours of incubation pointed out by Read does not arise when the buffer solution is used for collecting the stool sample.

6. When very large numbers of stool specimens of contacts have to be examined expeditiously, it is convenient to examine them in groups of ten. The contents of 10 specimen bottles are added to a flask containing the required amount of the fluid enrichment medium and a few loopfuls of the enriched culture plated after 18-20 hours' incubation on modified Riley's medium. Adequate care must be taken in pouring the contents to avoid contamination of the original bottles which are set aside until the results of the group are known. In the event of the isolation of *V. cholerae* from any group, the small remnant of the specimen contained in each of the bottles constituting the group is individually treated by enrichment and plating to determine which was contributory to the positive isolation of the group.

7. The colonies of *V. cholerae* have a characteristic appearance both on Aronson's and Riley's media. Portions of such colonies are picked out with a platinum needle and tested by slide agglutination against a suitable dilution (1/50 to 1/100) of a cholera agglutinating serum containing both Inaba and Ogawa anti-O agglutinins or preferably against two agglutinating sera, one for Inaba and the other for Ogawa. During an epidemic, it is generally adequate to base a provisional diagnosis of cholera on a positive agglutination obtained by this procedure pending confirmation by subsequent testing of cultures raised from colonies picked out by this screening.

8. For further steps in the identification of the isolated vibrio, a few colonies which give a positive slide agglutination test are subcultured on agar slants and in tubes of isotonic Douglas broth (tryptic digest). A suspension of an 18-to 24-hour growth of agar in 0.85% salt solution containing 0.2% formalin, adjusted to contain approximately 2,000 million organisms per ml (Brown's opacity tube No. 2), is used for serological identification and a 24-hour growth in Douglas broth for the haemolysis test.

9. Agglutination tests are carried out in a waterbath at 52° C with Inaba and Ogawa anti-O agglutinating sera. Young cultures of freshly isolated strains show a tendency to rapid lysis and, if a number of tests are being done at a time, it helps to place each rack in the waterbath as soon as the addition of the culture suspension has been made without waiting for the whole series of additions to be completed. The 'O' agglutination of cholera usually appears very early and a preliminary reading may be taken at the end of 2 hours, but the final reading is taken the next day,

after the racks have been stood at room temperature overnight. The suspensions should agglutinate at over 50%-75% of the titre of either Inaba or Ogawa anti-O serum.

10. The haemolysis test is done by adding 1 ml of 24-hour culture in isotonic Douglas broth to 1 ml of a 5% suspension of washed sheep or goat erythrocytes, incubating the mixture at 37° C for 2 hours followed by overnight storage in the cold room. It is essential to make sure that the erythrocytes used in the test are not fragile in 0.65% saline. Alternatively, a saline suspension of a 24-hour growth on nutrient agar, standardized to contain 8,000 million organisms per ml, may be used. To 1 ml of this vibrio suspension is added 1 ml of a 3% suspension of washed erythrocytes and the mixture treated as above. Krishnan, who recently made a detailed study of the various factors involved in the tests, has shown that more consistent results may be obtained by using broth cultures in preference to saline suspensions of agar cultures and that a 24-hour growth in broth is preferable to a 48- or 72-hour growth.

11. The culture in Douglas broth is also utilized for the inoculation of fermentation tubes for the determination of the range of action of the vibrio under test. *V. cholerae* produces acid without gas in glucose, mannose, saccharose, and maltose, but not in lactose and arabinose. Cholera red reaction can be demonstrated by the addition of a few drops of pure sulfuric acid to a 24-hour peptone-water culture of the organism, but the test is not distinctive of *V. cholerae*. Many other vibrios also give the reaction. The Voges-Proskauer reaction is negative.

12. Although speed in diagnosis of cholera is very important, it is equally essential that judgement should be reserved and only a provisional report returned until the essential tests for the identification of the *V. cholerae* are completed, more especially in areas where cholera is not usually prevalent. Under favourable conditions it is possible to offer a provisional diagnosis, by the procedures outlined, in 16-20 hours. A definitive diagnosis takes 48 hours more.

Appendix 1

Preserving Medium for the Transmission of Specimens

12.405 g boric acid (H_3BO_3) and 14.912 g potassium chloride (KCl) are dissolved in about 800 ml of hot distilled water. The solution is cooled and made up to 1 litre. From this stock solution 250 ml are taken, mixed with 133.5 ml of N/5 caustic soda, and the whole made up to 1 litre. 20 g dried sea salt (common salt from the bazaar serves equally well) are dissolved, and the buffer solution filtered through paper, dispensed in 10-ml quantities in 30-ml screw-capped bottles, and sterilized in the autoclave. The sterilized buffer solution has a pH of 9.2.

Appendix 2

A Modified Wilson and Riley's Medium for Plating

	ml
Douglas agar, pH 8.8 (melted and cooled to 50° C) . . .	100.0
20% sodium sulfite solution	4.8
Liquor bismuthi ⁵	0.16
Absolute alcohol	0.2
10% mannose solution	1.0

Mix and pour into plates.

Appendix 3

Read's Modification of Wilson and Blair's Medium

All ingredients are made up in separate solutions and kept in stoppered bottles. Mannose is made up in 10% solution for the requirement of the day and sterilized by boiling. Sodium sulfite is made up in 20% solution and slightly warmed to dissolve.

Formula of the medium :

	ml
2% peptone solution	8.8
Sea-salt mixture ⁶	1.2
Distilled water or stool suspension in distilled water . . .	10.0
10% mannose solution	1.0
Liquor bismuthi ⁵	0.12
20% sodium sulfite solution	1.2
Absolute alcohol	0.2
HgCl ₂ , 1/10,000 solution	0.8

adjusted to a pH of 9.2 with N caustic soda, with thymol blue as indicator.

⁵ According to Wilson & Blair (1931) Liquor bismuthi consists of :

bismuth citrate	60 g
ammonia (12.5%)	20 ml
distilled water, sufficient to make up to	500 ml

Liquor bismuthi is prepared as follows: a bottle with a ground-glass stopper is filled almost completely with 500 ml of distilled water and the liquid level is marked on the side of the bottle. The water is poured out, and 60 g of bismuth citrate introduced through a wide funnel, followed by 50 ml of distilled water. The citrate is mixed with the water into a smooth paste, using a glass stirrer. Next, 20 ml of 12.5% ammonia are added. The mixture is stirred with a glass rod and a chemical reaction takes place with evolution of heat. The glass stopper is inserted, the bottle shaken, and as soon as the bismuth citrate has almost entirely dissolved distilled water is added up to the 500 ml mark.

⁶ Formula of the sea-salt mixture :

	Parts
NaCl	27.00
KCl	1.00
MgCl ₂ , 6H ₂ O	3.00
MgSO ₄ , 7H ₂ O	1.75
Distilled water	100.00

Annex 2

RETROSPECTIVE DIAGNOSIS OF CHOLERA THROUGH STUDY OF AGGLUTININ RESPONSE FOLLOWING ANTICHOLERA INOCULATION¹

The agglutinin response to anticholera inoculation in three groups of individuals was studied: (1) normal group — persons who had not had cholera previously and had not received any anticholera inoculation; (2) inoculated group — persons who had not had cholera previously but had received one or more anticholera inoculations; and (3) cholera group — persons who had had cholera previously.

The object of the study was to determine the agglutinin level in these groups before and after inoculation with a known make and dose of cholera vaccine containing both Inaba and Ogawa vibrios, and to find out if the agglutinin response to anticholera inoculation was significantly different and enough to be used as an aid to retrospective diagnosis of cholera infection.

Previous workers have used two types of antigens for the agglutination test: suspensions of living agglutinable vibrios in saline or a heated suspension of vibrios. 74 comparative tests conducted with heated and unheated antigen and known positive sera showed that unheated antigen gave markedly better results than heated antigen. The latter not only gave a lower percentage of positive results (55%) but also gave positives in lower titres. Hence living vibrio suspensions were used as antigen. In the test the serum antigen mixtures were incubated at 52° C in a waterbath for 3 hours and then kept in the incubator overnight at 37° C. The anticholera vaccine used was in all cases the Kasauli vaccine. 1 ml of vaccine containing 8,000 million organisms was given in every case, and the blood for testing was obtained 14 days after inoculation.

So far a total of 175 persons have been examined; in the first group 18 persons, in the second group 132 persons, and in the third group 25 persons. The results obtained are given below:

Normal group. The sera of 18 persons were tested before inoculation. 14 failed to agglutinate both Inaba and Ogawa suspensions, even in a dilution of 1/10. 3 agglutinated in a dilution of 1/20, and 1 in 1/80. 16 of the 18 were inoculated with cholera vaccine, and a fortnight later their

¹ Note submitted by Dr. K. V. Krishnan, Professor of Microbiology, All-India Institute of Hygiene and Public Health, Calcutta, India; and Captain S. N. Dutta, Assistant Research Officer, Cholera Enquiry, All-India Institute of Hygiene and Public Health, Calcutta, India.

sera were re-tested: 2 gave negative results in a dilution of 1/10, 13 gave positive results in a dilution of 1/80 or less, and 1 gave a positive result in a dilution of 1/320.

Inoculated group. 132 persons were examined in this group. They gave a history of having had one or more anticholera inoculations previously. The date of receiving the last inoculation varied from a few months to a few years. Of the 132 persons examined, only 74 gave positive agglutination; the other 58 gave negative results in a dilution of 1/10. Of the 74 positive, 31 gave a titre of 1/20 or less, 41 gave a titre of 1/80 or less, and 2 gave a titre of 1/320 or less.

119 persons in this group were inoculated with 1 ml of cholera vaccine and their blood sera examined after a fortnight. Of these, 113 gave positive agglutination and 6 gave negative results. Of the positive, 11 gave a titre of 1/20 or less, 44 gave a titre of 1/80 or less, and 58 gave a titre of 1/320 or less.

Cholera group. The persons in this group were either convalescents from a recent attack of cholera or old recovered cases. There were 25 persons in this group; 17 of these had been bacteriologically diagnosed as cholera cases (either Inaba or Ogawa) and 8 had been diagnosed on clinical grounds (no bacteriological examination had been made, or bacteriological examination had given a negative result). Of the 8 bacteriologically unconfirmed cases, 4 gave negative agglutination in 1/10 and the other 4 gave positive in 1/80 or less. Of the 17 bacteriologically diagnosed cases, all gave positive agglutination: 4 gave positive in 1/80 or less, 9 gave positive in 1/320 or less, 3 gave positive in 1/1280 or less, and 1 gave positive in 1/2560.

TABLE I. AGGLUTININ LEVEL IN DIFFERENT GROUPS

	Number tested	Negative reaction	Positive reaction				
			1/20 or less	1/80 or less	1/320 or less	1/1280 or less	1/2560
Normal group { A . . .	18	14	3	1	—	—	—
B . . .	16	2	3	10	1	—	—
Inoculated group { A . . .	132	58	31	41	2	—	—
B . . .	119	6	11	44	58	—	—
Cholera group { A . . .	17	—	—	4	9	3	1
B . . .	4	—	—	—	1	3	—

A = before inoculation

B = after inoculation

Four of the cholera convalescents were inoculated with 1 ml of vaccine and their sera tested a fortnight later. The agglutinin response in 3 cases was positive over 1/320 and in 1 case it was positive in 1/160. As the work is incomplete no conclusions are drawn.

Inaba and Ogawa response

(a) *In vaccinated persons.* The agglutinin response to Inaba and Ogawa antigens in vaccinated persons following anticholera inoculation was studied. In a group of 26 people who had previously received one or more inoculations of cholera vaccine, 7 gave the same level of response for Inaba and Ogawa, 13 a higher response for Inaba than for Ogawa, and 6 a higher response for Ogawa than for Inaba. These people were examined again after re-vaccination: 13 gave the same response for Inaba and Ogawa, 8 a higher response for Inaba than for Ogawa, and 5 a higher response for Ogawa than for Inaba. It appears from this that the mixed vaccine used in India elicits an equal response to both Inaba and Ogawa.

TABLE II. RESPONSE TO INABA AND OGAWA ANTIGENS

	Number tested	Level of response		
		Same for Inaba as Ogawa	Higher for Inaba than Ogawa	Higher for Ogawa than Inaba
Vaccinated group:				
Before inoculation . . .	26	7	13	6
After inoculation . . .	26	13	8	5
Cholera cases:				
Inaba	3	3	—	—
Ogawa	10	3	3	4

(b) *In cholera cases.* 3 cases of cholera bacteriologically diagnosed as Inaba were tested. Their sera agglutinated Ogawa to about the same level as Inaba. Of 10 Ogawa cases studied, 4 gave distinctly higher titres for Ogawa than for Inaba, 3 gave the same titre for Inaba as for Ogawa, and 3 gave a slightly higher titre for Inaba than for Ogawa.

In 1 Inaba and 3 Ogawa cases, second samples were obtained about the fourth week of convalescence and examined. In all cases the agglutinin titre obtained was about the same as the titre for the first sample taken at the end of the second week of convalescence.

Annex 3

R TO S VARIATION OF *V. cholerae* KOREIN, IN PRESENCE OF ANTIPHAGE SERUMPreliminary Note¹

Antibacteriophage serum was prepared by intravenous injection of cholera phage in rabbits. Four doses were injected, starting with 0.5 ml and increasing the doses up to 2 ml. The intervals between the doses were three, five, and seven days, and ten days after the last injection the serum was collected.

The activity of the cholera phage on the *V. cholerae* Korein was one in ten milliards. The number of phage corpuscles was estimated by diluting the phage in broth and plating out 0.05 ml of it on 1.2% alkaline agar plates inoculated with vibrios. This number varied between 10 and 20 milliards per ml.

The antiphage serum showed a weak agglutinating power on the Korein vibrios, its agglutinating titre being about 1/300. An antiphage serum dilution 1/500 was made in broth and 2 ml were put in each of 5 tubes adding to each tube 2 ml of a phage dilution, making a final serum dilution of 1/1000.

TABLE I. ACTIVITY OF ANTIPHAGE SERUM ON VARIOUS DILUTIONS OF *V. CHOLERAE* KOREIN PHAGE

	Dilution of phage		Serum (antiphage) 1/500	Phage activity after 4-hours' incubation
1	10 ⁻⁴	2 ml	2 ml	+
2	10 ⁻⁵	2 ml	2 ml	—
3	10 ⁻⁶	2 ml	2 ml	—
4	10 ⁻⁷	2 ml	2 ml	—
5	10 ⁻⁸	2 ml	2 ml	—

After incubation at 37° C for four hours, 0.05 ml of each phage-serum mixture was spread out on agar-plates (1.2% agar), and then inoculated with *V. cholerae* Korein.

After 24 hours at 37° C, phage-activity was present on only one plate, showing three bare spots (2 ml of phage 10⁻⁴ + 2 ml serum).

On plating out the original dilutions (not containing the antiphage) 10⁻⁶, 10⁻⁷, and 10⁻⁸, a huge number of bare spots developed, the centre of the

¹ Note submitted by Dr. W. Doorenbos and Dr. G. N. Cossery Bey, Central Laboratories, Ministry of Public Health, Cairo, Egypt.

agar plates being completely bare of growth. Counting phage-corpuscles by dilution and plating out gives variable results, but in the above experiment a definite inhibition of phage-activity was noted after 4 hours' incubation in presence of antiphage serum 1/1000.

R to S Transformation

From a five days' old secondary growth of phage in broth of *V. cholerae* Korein an R-variant was obtained and the effect of the phage-serum on this vibrio was tested.

Serum dilutions 1/100 and 1/500 in broth were inoculated with the R-variant; a broth culture with 1/500 normal rabbit serum inoculated with this R-variant served as control.

After 48 hours' incubation a loop from each culture was transferred to similar tubes of broth serum, and after another 48 hours this was repeated. After incubation for 72 hours the cultures were plated out on cholera-agar plates.

After 24 hours' incubation there was no apparent difference between the R-vibrios grown in presence of antiphage serum and those grown in normal rabbit serum, the colonies being small and dry, while it was impossible to emulsify the vibrios in normal saline.

The plates were left at room temperature for five days when the colonies on the control plates were found to keep their R-appearance; while the vibrios grown repeatedly in the presence of phage-serum showed a definite tendency to revert to the S-type.

The colonies had increased in size, the centre of the growth remaining R but the edges having an S-appearance. 60%-70% of the colonies showed this phenomenon and there was no apparent difference between the plate inoculated from the 1/100 serum dilution and the 1/500 serum dilution.

A transfer on cholera-agar tubes was made from the S edges of several colonies from both (1/100 and 1/500 serum dilutions) plates; the growth obtained was smooth in appearance and could be emulsified easily in normal saline.

The agglutinability of these S-vibrios was the same (1/3200) as that of the original S growth of this vibrio, before being submitted to phage-action.

Discussion

The antiphage serum showed a definite inhibiting power on the phage activity, but it contained also some agglutinins against the vibrios.

The R to S transformation of the vibrios in presence of this serum may be due to the fact that it is an "immune serum", or to its antiphage properties, or to a combination of both factors.

The fact that the agglutination titre of this serum is low, 1/300, giving a complete clarification in 1/300 and not in 1/500, and the fact that dilutions 1/100 and 1/500 had the same effect on the R to S transformation, make us tend to believe that the antiphage property has been the primary cause of the transformation. The R-vibrios grown in presence of normal rabbit serum 1/100 did not show this phenomenon.

The possibility, although remote, of a spontaneous R to S transformation of the R-vibrios when grown on different media had to be taken into consideration ; but the fact remained, as shown in the above-mentioned experiments, that under the influence of a specific antiphage an R-vibrio reverted to its original S-form.

Further research on this subject is being done and results will be reported in due course.

The authors wish to thank Sir Aly Shousha, Pasha, formerly Under-Secretary of State, Ministry of Public Health, Egypt, at whose suggestions and by whose encouragement this work was undertaken.

Annex 4**PERSONS PRESENT AT THE JOINT MEETING OF THE OIHP/
WHO STUDY-GROUP ON CHOLERA, AND THE CHOLERA
ADVISORY COMMITTEE OF THE INDIAN RESEARCH FUND
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